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PURIFICATION OF TOXIN FROM *E.* COLI ISOLATE Pe68 BY ION-EXCHANGE CHROMATOGRAPHY

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Abstract: *E. coli isolate pe68 which showed positive to hlyA toxin was subjected to protein purification by Ion-exchange chromatography. The purification steps involed salting out, dialysis and ion-exchange separation carried out in column packed with ion-exchanger. In the present study Sephrose 6B as fast-flow weak anion and Carboxymethyl Cellulose as fast-flow weak cation was used as stationary phase and elution buffer was used as mobile phase. Different concentrations of Tris HCl and Sodium Chloride solution i.e., 1M of sodium chloride solution and 1M Tris HCl buffer with pH 7 was used as elution buffer. Different fractions of elution were collected every 5 minutes and were subjected to protein estimation by Lowry's method. Protein obtained from above purification steps were subjected to molecular weight determination by SDS-PAGE using low-protein molecular weight markers. SDS-PAGE of the protein sample resulted in a single band close to the protein marker with 33 kDa.*

Keywords: *Escherichia coli, ion-exchanger chromatography, SDS-PAGE*

1. INTRODUCTION

Escherichia coli belongs to family *Enterobacteria.* Cells are rod shaped, motile with peritrichous flagella arrangements, gram negative, facultative anaerobic bacteria found in lower intestine of human and animals that grows at pH 7, temperature 37ºC. *Escherichia coli* can grow aerobic and anaerobic respiration. *E. coli* is found in food, water, fresh vegetables, uncooked meat, raw milk etc. It comes in contact with human or animals through contact with contaminated water. It is widespread intestinal parasites of mammals and birds. The toxins produced by *Escherichia coli* are also known as Shiga-toxins or verotoxin. Shiga-toxins (Stx) are produced by some strains of *Escherichia coli* belonging to coliform group. The bacteria are acquired by ingestion during the first few days of newborn. *E. coli* has the abilty to kill Vero cells. The toxins produced by *Escherichia coli* include heat-labile and heat-stable enterotoxin causing lower fever, nausea, vomiting, stomach cramp and bloody diarrhea. These toxins are responsible for bloody diarrhea causing haemolytic uremic syndrome in humans.

The infection spreads from one person to other persons. *Escherichia coli* can survive for months in soil and water. It is killed at 60ºC in 20 minutes and chlorine at a concentration of 0.5 to 1 part per million.

Escherichia coli is a common contaminant in most of the foods. With the importance of pathogenicity caused by its toxins it is necessary to purifiy and characterize the toxins produced by *E. coli* from food sources. Increased awareness on the benefits and strict observance of personal hygiene by the population are the best preventive measures against such bacteria agents in the face of increasing antimicrobial resistances. *Escherichia coli* grows on simple media. *E. coli* strains isolated from panipuri samples showed green metallic sheen on Eosin Methylene Blue agar.

Using multiplex PCR Maldonado *et al.* examined the presence of virulence gene markers (*stx*$_1$, *stx*$_2$, *eaeA*, *hlyA*) in 1,698 environmental isolates of *E. coli* and 81 isolates from food and clinical sources [1]. The PCR analysis showed that ~5% (79 of 1,698) of the total environmental isolates and 96% (79 of 81) of the food and clinical isolates were positive for at least one of the genes. Of the food and clinical isolates, 84% (68 of 81 isolates) were positive for all four genes. Of the subset of environmental isolates chosen for further analysis, 16% (13 of 79 isolates) were positive for *stx*$_2$ and 84% (66 of 79 isolates) were positive for *eaeA*; 16 of the latter strains were also positive for *hlyA*.
Ion-exchange chromatography is a purification process that is used to separate ions based on their charges. It is commonly used in protein purification.

Sephrose 6B which is "Fast flow weak anion" and a polysaccharider polymer was used as packing material for anion exchanger which equilibates the viscous of buffer at reduced flow rate helping in purification of sample. Carboxymethyl Cellulose (CMC) or Cellulose gum a "Fast-flow weak cation" was used as stationary phase for cation exchanger which acts as a viscosity modifier or thinener. Based on their net-charges the protein was seperated by ion-exchange chromatography. Different concentration of Tris HCl and Sodium solution was used as elution buffer which removes the undesired protein and other impurities and elute the proteins of interest. Lowry's method for total protein estimation is most commonly performed assay for calorimetric procedure. This method is sensitive because it develops colour forming reaction. Combination of copper sulphate solution and the protein, a blue colour is produced which can be measured at 660 nm absorbance. SDS-PAGE is a common procedure for protein separation by electrophoresis utilize the sodium dodecyl sulphate to denature the protein and separates by the protein sizes.

Brien O' and Laveck purified a toxin from an entero pathogenic strain of *Escherichia coli* (*E. coli* H30) to apparent homogeneity from cell lysates [2]. The steps used to isolate the *E. coli* H30 toxin included French pressure-cell disruption of bacteria grown in iron-depleted media followed by Affi-Gel blue chromatography, chromato-focusing and anti-Shiga toxin affinity chromatography. The mobilities of the subunits of radio iodinated *E. coli* H30 toxin and Shiga toxin were observed after the two toxins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The toxins were found to be identical. *E coli* contamination particularly among the lower income groups. Street foods specially the panipuri on the roadside attracts the people based on taste and low cost. Since it is readly available. It is recognized that street foods play a vital role in food contamination. Aiming at the purification of protein toxin from *E. coli* isolated from panipuri the present study was designed.

2. MATERIALS AND METHODS

2.1 E. Coli CULTURE

E. coli isolate pe68 which showed positive to *hlyA* during PCR analysis was inoculated onto Eosin Methylene Blue (EMB) agar media and was incubated at 37ºC for 24 hr. The culture was maintained for further studies.

2.2 PARTIAL PURIFICATION OF PROTEIN

2.2.1 AMMONIUM SULPHATE PRECIPATION

E. Coli was inculated into 200 ml of nutrient broth and incubated for 24 hr at 37ºC. The overnight-incubated culture was centrifuged at 10,000 rpm for 15 mins. Supernatant was subjected to ammonium sulphate precipitation. To the supernatant solid ammonium sulphate was added to 40% saturation and mixture was stirred at 4ºC for 2 h and centrifuged at 25,000 rpm for 25 min at 4ºC. Supernatant was discarded and the pellets was subjected to 60% saturation of ammonium sulphate with continuous stirring for 2 hrs at 4ºC and centrifuged at 15,000 rpm for 25 min at 4ºC. The second precipitate was collected by centrifugation and was dissolved in 20 ml of phosphate buffer saline with pH 7.

2.2.2 DIALYSIS

Sample was then subjected to dialysis. Dialysis tubes, which was previously soaked in 1 M Tris HCl buffer, was used for dialysis of the precipitate. Sample was kept for dialysis in 1% sucrose at 4ºC for 18 hrs [3].

2.3 ION-EXCHANGE CHROMATOGRAPHY

Protein sample after dialysis was subjected to both anion exchange and cation exchange chromatography.

2.3.1 ANION CHROMATOGRAPHY

Dialyzed pooled material with 20 mg of protein was applied to 145 mm length 15 mm radius column filled with Sephrose 6B gel. The column was washed with wash buffer. Different concentrations of 1M Tris-HCl and 1M Sodium Chloride solution with pH 7.0 as elution buffer was added every 5 mintues. Elution collected every 5 minutes was subjected to protein estimation [4]. Elution of protein was monitored at 660nm.

2.3.2 CATION CHROMATOGRAPHY

Fraction with highest protein content from anion-exchange chromatography was applied to column packed with Carboxymethyl Cellulose. The column was washed with wash buffer. Different concentrations of 1M Tris-HCl and 1M Sodium Chloride solution with pH 7.0 as elution buffer was added every 5 mintues. Elution collected every 5 minutes was subjected to protein estimation [4]. Elution was monitored at 660nm.

2.4 ESTIMATION OF PROTEIN

Elutions obtained from ion-exchange chromatography after purification was subjected to protein estimation by Lowry's method [5]. A stock solution of 1 mg/ml concentration of Bovine serum albumin was used as a standard compound. Elution of protein was monitored at 660 nm. Fraction with highest concentration of protein was subjected to SDS-PAGE using molecular weight marker.

2.5 SDS-PAGE

Cell free supernatant, ammonium sulphate precipitated sample, sample after dialysis and sample after column chromatography were subjected to SDS-PAGE along with the markers. 250 µl of protein sample from each step of the purification procedure was mixed with 5 µl of sample buffer (1M Tris hydrochloride (pH 6.8), 12% sodium dodecyl sulphate (SDS), 5.0% 2-mercaptoethanol, 10% glycerol, 1% bromophenol blue) and heated at 75ºC for 10 min. 10 µl of the mixture was loaded onto individual lanes of 7.5% stacking gels with pH 6.8 (0.75 mm thick). Electrophoresis was carried out at a constant voltage of 100v until the stacking dye reaches the top of the resolving gel (15%) and then at 150v until the dye front reaches the bottom of the resolving gel. Gel was fixed with fixing solution and stained with Coomassie brilliant blue for 3 hrs. Gel was destained using methanol-water containing 10% Glacial acetic acid for overnight. Finally, the gel was observed under UV illuminator for bands. A blue band on gel indicated the presence of protein [6].

3. RESULTS

E. coli on Eosin Methylene Blue agar showed nucleated colonies with green metallic sheen "Fig 1". Partial purification and concentration step by ammonium sulphate precipitation followed by dialysis showed multiple protein bands "Fig 2".

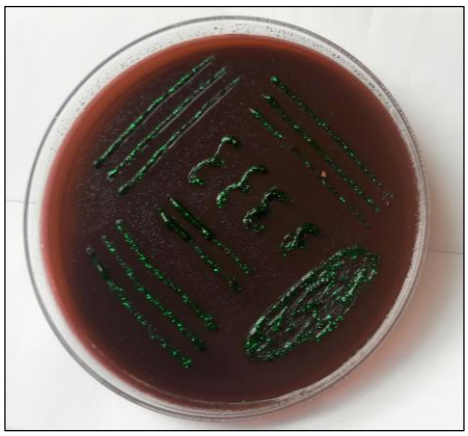

Figure 1: Growth of isolate pe68 on Eosin Methylene Blue agar medium

Among the protein fractions collected from purification by ion-exchange chromatography (anion and cation), fractions that showed highest protein concentration by Lowry's method were selected for further studies "TABLE 1". Fraction number 6 for anion exchange chromatography with 2.33 mg/ml of protein and fraction number 4 for cation exchange chromatography with 1.56 mg/ml of protein were subjected to SDS-PAGE with protein marker. Staining with Coomassie blue after electrophoresis showed prominent band with molecular weight of 33 kDa "Fig 3". This confirmed the sample purity.

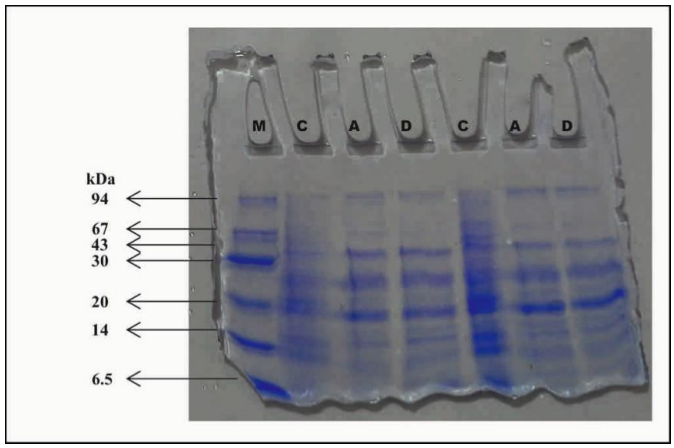

Figure 2: SDS-PAGE of protein from pe68 after partial purification

M-Molecular weight marker, C-Cell free supernatant, A-Ammonium Sulphate precipitation, D-dialysis

Table I: Concentration of protein in fractions of column chromatography

Elution (1ml)	Volume of distilled water (ml)	Volume of alkaline copper reagent (ml)	Incubate for 10 mins at room temperature	Folin reagent (ml)	Incubate for 30 mins at room temperature	OD at 660 nm	mg/ml
Blank	0.9	5		**0.1**		----	----
4	0.9	5		**0.1**		0.125 (cation)	1.56
6	0.9	5		**0.1**		0.204 (anion)	2.33

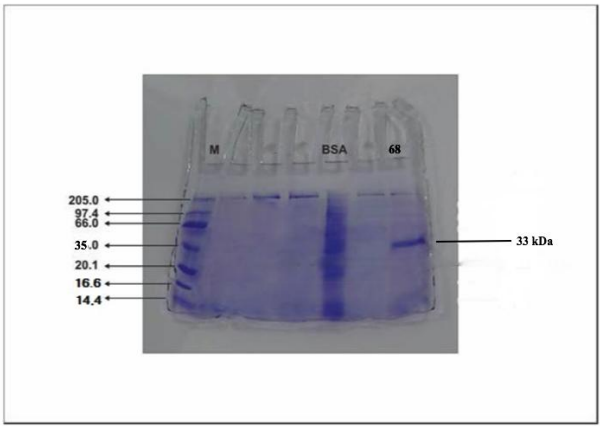

Figure 3: SDS-PAGE of protein from isolate pe68 after ion-exchange chromatography
M-Molecular weight marker, BSA-Bovine Albumin Serum

4. DISCUSSION

The initial fraction with multiple bands of protein proved that some impurities were present in the sample. As the purification method progressed numbers of bands were diminished and the prominence of protein was visualized. Smaller size proteins move vigorously than the larger ones. Bands were visualized after staining the gel with Coomassie blue dye. Even smaller quantity as 0.1µg of protein when stained with Coomassie blue gives a distinct band. The ion exchange chromatography showed purity of protein in the sample. David *et al.* have carried out purification of *Escherichia coli* Shiga-like toxin II variant [4].

5. CONCLUSION

The purity of protein was seen as the toxin revealed a single band. Ion-exchange chromatography is more efficient than other chromatography. It can be widely used for commercial purposes. SDS-PAGE requires small amount of sample. It is a useful method for separating and characterizing protein. Molecular weight of protein can be determined.

6. ACKNOWLEDGMENTS

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